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Regulation of cysteine-rich intestinal protein by dexamethasone in the neonatal rat

(LIM motif/zinc finger/developmental regulation/zinc binding)

CATHY W. LEVENSON*†, NEIL F. SHAY*, LINDA M. LEE-AMBROSE*, AND ROBERT J. COUSINS*‡

*Department of Food Science and Human Nutrition, Center for Nutritional Sciences, University of Florida, Gainesville, FL 32611; and †Department of Medicine, Committee on Human Nutrition and Nutritional Biology, University of Chicago, Chicago, IL 60637

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ABSTRACT **The cysteine-rich intestinal protein (CRIP) is an intestinal zinc-binding protein containing a single copy of a cysteine-rich domain known as the LIM motif. CRIP mRNA and protein levels increased in the rat small intestine throughout the suckling period, reaching highest levels by the late weanling stage. A similar developmental pattern of CRIP protein levels was also detected by an increase in zinc binding to CRIP-containing HPLC fractions of intestinal cytosol. Administration of the synthetic glucocorticoid hormone dexamethasone to neonates caused the precocious rise of CRIP mRNA and protein. In adult rats, CRIP mRNA levels were not significantly altered by dexamethasone. Maximal CRIP mRNA content was detected in cells from the mid-villus, as confirmed by expression of cryptdin mRNA. In this report we show the glucocorticoid regulation of the LIM motif-containing protein CRIP and suggest that glucocorticoid hormones play a role in developmental regulation of CRIP.**

The cysteine-rich intestinal protein (CRIP) was first identified as an intestinal mRNA induced just before weaning (1). More recently, CRIP was shown to be a zinc-binding protein in rat intestine (2) and has been proposed to function as a transcellular carrier of zinc in intestinal cells (3).

CRIP is unique in that it contains a single copy of a cysteine-rich domain known as the LIM motif. The LIM motif, Cys-Xaa_2-Cys-Xaa_{17-19}-His-Xaa_2-Cys-Xaa_2-Cys-Xaa_2-Cys-Xaa_{7-11}-(Cys)-Xaa_8-Cys, was first identified in the *lin-11*, *isl-1*, and *mec-3* genes and was named for these genes (4). In these three genes, and others since reported (5–7), the LIM motif is present as a two-copy tandem of the LIM amino acid sequence described above. It has been proposed that the cysteine-rich domain of CRIP is in a double zinc-finger arrangement (7). The significance of a single copy of the LIM motif vs. a tandem array is not yet known, nor is it known whether CRIP interacts with itself or other proteins to form dimers or higher-ordered complexes that would produce the functional equivalent of the tandem LIM motif. Many of the tandem LIM motif proteins have homeodomains and, therefore, proposed roles as transcription factors (8). It is unknown whether CRIP, which lacks a homeodomain, plays any role in the regulation of genes or serves another zinc-requiring cellular function.

Given the developmental regulation of CRIP (1) and that glucocorticoid hormones have been shown to play a role in the normal development of the neonatal small intestine (9), we have examined the regulation of CRIP mRNA levels by the synthetic glucocorticoid, dexamethasone. Here we present evidence of hormonal regulation of this LIM motif-containing protein and its mRNA and demonstrate an increase in ^{65}Zn binding to CRIP during neonatal development.

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EXPERIMENTAL PROCEDURES

Animals. Sprague–Dawley rats (Harlan–Sprague–Dawley, Indianapolis) were used in all experiments. Pregnant dams were received on day 14 of gestation, and adult males weighed 150–200 g. Neonatal animals were a random mixture of males and females. A commercial diet (Rodent Laboratory Chow no. 5001, Ralston-Purina, Saint Louis) and water were provided ad libitum. All animal experiments were approved by the institutional animal care committee.

Hormone Treatment. On postnatal days 1 and 2 (where day 0 was day of birth) pups from separate litters, born on different days, were randomly selected for s.c. injection with dexamethasone (Dex; 1 mg/kg of body weight) or the vehicle (5 mM NaOH in saline) (10, 11). Control animals were vehicle-treated littermates. On days 1 (without treatment), 3, 7, 14, and 21 (n = 3), RNA was purified from small intestine for RNA analysis. Adult male rats were also injected i.p. with Dex (1 or 2 mg/kg). After anesthetization with methoxyfluorane, intestinal samples (n = 2–4) were taken 3, 6, and 12 hr after injection for isolation of RNA.

Isolation of Enterocytes. Enterocytes were sequentially isolated from the villus to crypt of the jejunum by using a modification of the method of Weiser (12). A 20-cm section of jejunum (10 cm from stomach) was first rinsed with 0.154 M NaCl/1 mM dithiothreitol and then filled with phosphate-buffered saline (PBS)/1.5 mM EDTA/0.5 mM dithiothreitol. Both ends of the intestinal loop were clamped, and the entire loop was incubated in PBS at 37°C. After each 5-min incubation period, the intestinal loop was drained and rinsed once with the incubation solution. Cells from each fraction were collected by centrifugation for 5 min at 200 × g. Cells were resuspended and homogenized in guanidinium isothiocyanate for isolation of RNA.

Northern Analysis. Total cellular RNA was immediately extracted from fresh intestinal samples with the guanidinium isothiocyanate method (13). RNA samples were subjected to agarose gel electrophoresis (1% wt/vol)/0.66 M formaldehyde. Gels were loaded with 15 μg of total RNA per lane. RNA was stained with ethidium bromide (added before electrophoresis) to verify the integrity of the RNA. Capillary blotting was used to transfer RNA to nylon membranes (GeneScreen, NEN). After UV crosslinking, blots were probed with a random-primed ^{32}P-labeled rat CRIP cDNA, metallothionein (MT) cDNA (14) or cryptdin cDNA (15). Equal loading of lanes was confirmed by visualization of ethidium bromide staining and by hybridization to a ^{32}P-labeled rat β-actin cDNA. Filters were autoradiographed at −70°C. Densitometry of the autoradiographs and peak area integration provided relative quantification of the amount of bound cDNA probe.

Abbreviations: CRIP, cysteine-rich intestinal protein; Dex, dexamethasone; MT, metallothionein.

‡To whom reprint requests should be addressed.

PCR. A CRIP cDNA fragment was synthesized using 5'- and 3'-PCR primers with restriction enzyme sites included in the primers to aid in cloning of the PCR fragment. Two primers were synthesized by the University of Florida DNA Synthesis Core Facility: 5'-GGATCCGCCGGCCTCGA-GTCCAGAGCCTACAACCTA-3' (5' end, bases 1–18) and 5'-GGTACCAAGCTTGCGGCCGCTGCTGTCTAGGGA-CAAGG-3' (3' end, bases 342–359). Two micrograms of rat intestinal mRNA was reverse-transcribed by using a first-strand reaction kit and the protocol supplied by the manufacturer (Invitrogen, San Diego). After extraction and precipitation, the reaction mixture was resuspended in 50 μl of 10 mM Tris·HCl/1 mM EDTA, pH 7.4. Ten microliters of this solution was included in a 100-μl vol containing deoxynucleotides (0.1 mM each), primers, and *Taq* polymerase with 50 mM KCl, 10 mM Tris (pH 9.0), 0.01% Triton X-100, and 1.5 mM $MgCl_2$. The reaction was subjected to 30 cycles of 60 sec at 94°C, 30 sec at 55°C, and 2 min at 72°C. After synthesis of the PCR product was confirmed by electrophoresis, it was digested with *Bam*HI and *Hin*dIII and ligated into *Bam*HI and *Hin*dIII-cut pGEM 3Zf(+).

^{65}Zn Binding to CRIP. The effect of age on ^{65}Zn binding by CRIP was determined by a modification of a described method (2, 3). Briefly, the entire small intestine (from stomach through ileum) was excised from untreated pups 7, 14, and 21 days after birth (n = 2). Intestines were flushed with saline to remove luminal contents, filled with 3.7 MBq of ^{65}Zn in 5 μM $ZnSO_4$, and incubated in PBS at 37°C for 15 min. A supernatant (referred to hereinafter as cytosol) was prepared by homogenation of tissue in 3 vol of ice-cold buffer (10 mM Tris·HCl, pH 8.6/154 mM NaCl/0.2 mM phenylmethylsulfonyl fluoride/leupeptin at 0.06 μg/ml/pepstatin A at 0.09 μg/ml. The homogenate was centrifuged at 40,000 × g for 30 min at 4°C. The cytosol was filtered (0.2 μm) and applied (200 μl, 12.5 mg of protein/ml) to a Superdex 75 HR 10/30 fast protein liquid chromatography column (Pharmacia/LKB) to separate cytosolic components by molecular size. Proteins were eluted from the column with 10 mM Tris·HCl/154 mM NaCl/10 mM $MgSO_4$ and collected in 0.5-ml fractions. ^{65}Zn was measured in HPLC fractions to determine ^{65}Zn binding to fractions previously shown to contain MT and CRIP (2, 3). MT concentrations were determined by Cd–hemoglobin affinity assay (16).

Metal-Binding Assay. A ^{65}Zn-binding assay was used to determine the effect of Dex treatment on the abundance of CRIP in the intestine of 3- and 7-day-old pups. Intestinal cytosols from Dex and vehicle-treated pups were prepared and separated by gel-filtration HPLC, as described above. Chromatographic fractions were transferred to 0.2-μm nitrocellulose membranes (Schleicher & Schuell) by slot blot. Nitrocellulose was equilibrated in 10 mM Tris·HCl, pH 7.5/1 mM $MnCl_2$/10 mM 2-mercaptoethanol (TMM) for 2 hr. The membrane was then incubated for 15 min in 50 ml of TMM-containing 18.5 MBq of ^{65}Zn and then washed with 10 mM Tris·HCl, pH 7.5 (2). In this method no binding of ^{65}Zn to MT is expected due to the presence of 2-mercaptoethanol (2). Membranes were autoradiographed at −70°C. Densitometry of the autoradiographs and peak area integration provided relative abundance of bound ^{65}Zn.

RESULTS

High levels of CRIP mRNA in the rat small intestine and smaller amounts in other organs such as colon, lung, spleen, adrenal, and testis have been reported (1, 7). We have verified the relative abundance and tissue specificity of CRIP expression (Fig. 1). We also detected CRIP mRNA in the cecum (≈76% of small intestine), skin (21%), heart (14%), skeletal muscle (5%), and stomach (5%) (Fig. 1). We found no

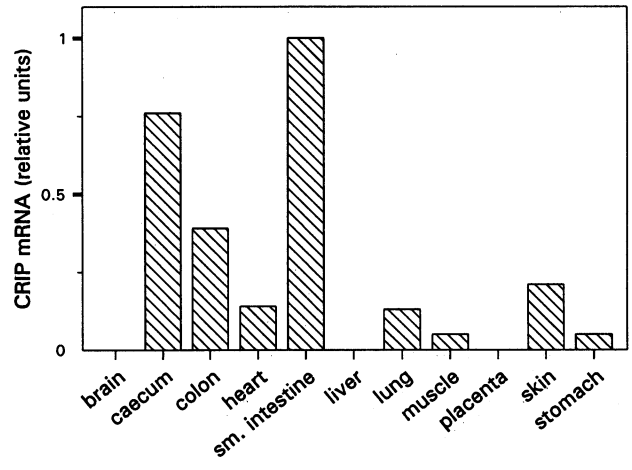

FIG. 1. Tissue distribution of CRIP mRNA. Bar graph of relative CRIP mRNA levels of selected organs as determined by Northern analysis. mRNA levels are expressed relative to levels in small (sm.) intestine. Total RNA (15 μg/lane) was separated by agarose gel electrophoresis, transferred to a nylon filter, and hybridized with a ^{32}P-labeled CRIP cDNA probe.

CRIP mRNA detectable by Northern analysis in the rat brain, kidney, or day 19 placenta (Fig. 1).

By RNA analysis, expression of CRIP mRNA along the villus-to-crypt axis of the jejunum was compared with that of β-actin mRNA and cryptdin mRNA. Cryptdin has been shown to be expressed exclusively in the cells of the crypt region of the small intestine (15). Of the seven intestinal fractions collected, only fractions 6 and 7, representing the cells predominately from the crypt region, were positive for cryptdin (Fig. 2). β-Actin mRNA levels were higher in fractions from the crypt region than from the villar regions. Relative CRIP mRNA levels were highest in fractions 4 and 5, the mid-villar region (Fig. 2).

CRIP mRNA levels were detectable by RNA analysis in the rat small intestine in the late gestational period and increased throughout the suckling period, reaching highest levels around postnatal day 21 (Fig. 3). Although it has been reported (1) that CRIP mRNA levels decrease by 40% after day 24, our data show that CRIP mRNA levels in the 21-day-old rat intestine are approximately the same as in the adult rat. Binding of ^{65}Zn to CRIP-containing HPLC (Superdex 75 HR) fractions also increased with age (Fig. 4). Fig. 4 (*Top*) shows ^{65}Zn binding to intestinal proteins after the 15-min incubation of a 7-day-old rat intestine with radioisotope. On day 7, ^{65}Zn binding to MT was substantial; binding to CRIP appeared only as a small shoulder of the MT peak. By day 14, the MT peak had decreased significantly, and a separate CRIP peak (2) was evident (Fig. 4, *Middle*). On day

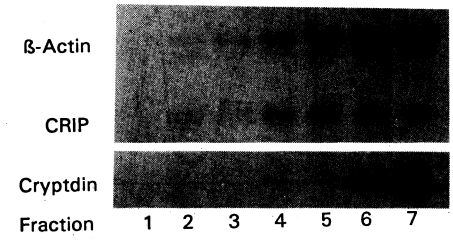

FIG. 2. Northern analysis of intestinal fractions. Enterocytes from proximal jejunum were sequentially isolated from villus to crypt. Fraction 1 represents the most villar fraction and nearest the lumen, whereas fraction 7 was taken from the crypt region. Total RNA (15 μg/lane) from each fraction was separated for Northern analysis. Blots were hybridized with ^{32}P-labeled β-actin, CRIP, and cryptdin cDNA probes.

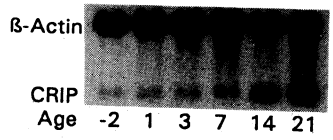

FIG. 3. Northern analysis of CRIP mRNA in developing rat intestine. The developmental increase in CRIP mRNA is shown by Northern analysis of small intestine on day 19 of gestation and postnatal days 1, 3, 7, 14, and 21. Total RNA (15 μg/lane) was separated by agarose gel electrophoresis, transferred to a nylon filter, and hybridized with ^{32}P-labeled CRIP and β-actin cDNA probes.

21, no zinc binding to MT was detected. However, there was a large increase in the binding of ^{65}Zn to CRIP on day 21 (Fig. 4). The fall in intestinal MT was consistent with a reduction in MT mRNA during the same period (Fig. 5).

When Dex was administered on postnatal days 1 and 2, the normal developmental expression of CRIP mRNA was altered (Fig. 6). Dex treatment transiently increased CRIP mRNA levels ≈2-fold (over vehicle-treated animals) on day 3. After day 3, CRIP mRNA levels of Dex-treated animals were not significantly different from vehicle-treated controls.

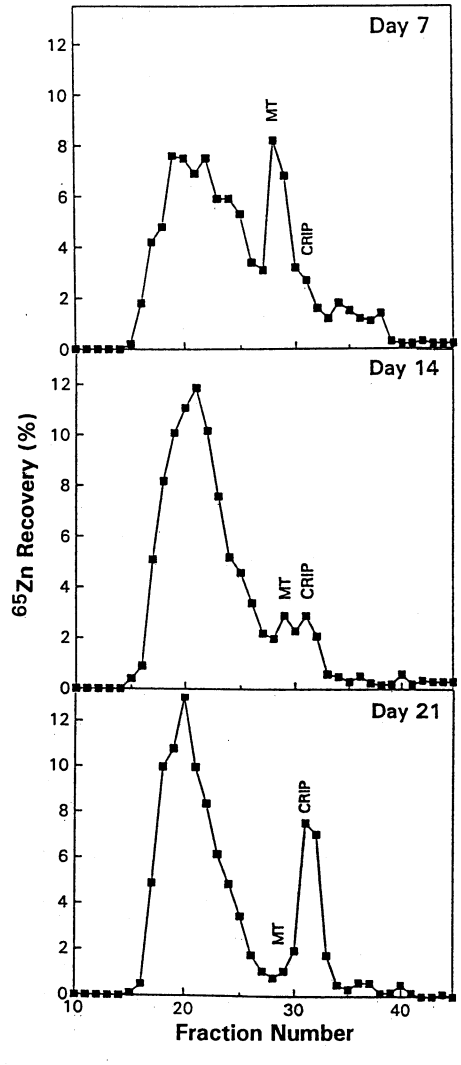

FIG. 4. Distribution of ^{65}Zn in cytosolic constituents of developing rat intestine. Intestinal mucosa from 7- (*Top*), 14- (*Middle*), and 21- (*Bottom*) day-old rats was labeled by incubation (15 min) of a ligated intestinal segment filled with ^{65}Zn. Intestinal cytosolic proteins were then separated into 0.5-ml fractions by Superdex 75 HR gel-filtration HPLC, and ^{65}Zn was measured in each fraction. Elution characteristics of MT and CRIP are shown.

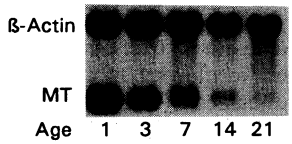

FIG. 5. Northern analysis of MT mRNA in developing rat intestine. The developmental decrease in MT mRNA is shown by Northern analysis of small intestine on postnatal days 1, 3, 7, 14, and 21. Total RNA (15 μg/lane) was separated by agarose gel electrophoresis, and blots were hybridized with ^{32}P-labeled MT and β-actin cDNA probes.

Binding of ^{65}Zn to CRIP-containing HPLC fractions (immobilized on nitrocellulose) isolated from intestine of 3-day-old rats was also increased 2-fold in Dex-treated animals as compared with vehicle-treated controls (Fig. 7). A similar increase in zinc binding was seen in Dex-treated 7-day-old pups (data not shown). In adult animals, no significant changes were seen in intestinal CRIP mRNA at 3, 6, 12, or 24 hr after treatment with 1 or 2 mg of Dex per kg (data not shown).

DISCUSSION

CRIP mRNA levels were found to be highest in the cells of the mid-villar region (fractions 4 and 5, Fig. 2). β-Actin and cryptdin cDNA probes were used as controls in RNA analysis to confirm the sequential nature of our fractions. Cryptdin is known to be expressed exclusively in the crypt cells (15). Our results demonstrate through cryptdin expression that fractions 6 and 7 (Fig. 2) represent cells from the crypt region of the intestine. β-Actin mRNA abundance increased gradually from villar to crypt fractions. This pattern of actin hybridization is likely due to more active cell division in the crypt regions. The finding that CRIP mRNA is predominately found in the mid-villar region is consistent with the synthesis of CRIP as the enterocytes migrate along the villus and with degradation of the RNA as the cells reach the villar tip. This pattern also is consistent with the proposed function of CRIP (2, 3) as a transcellular carrier of zinc, which would presumably be most highly expressed in the villar, rather than crypt, regions.

We and others (1) have demonstrated that CRIP mRNA levels increase in the rat intestine during the developmental period (Fig. 3). Our ^{65}Zn incorporation data suggests that the observed increases in CRIP mRNA during development are associated with increases in CRIP protein levels. Although CRIP levels are increasing in the normal developing neonatal rat, MT (Fig. 4) and MT mRNA (Fig. 5) levels are falling. It is possible that in the early neonatal intestine MT acts as a zinc reservoir for use until more mature zinc transport systems are fully operative by the time of weaning.

Developmental increases in glucocorticoid hormones may play a role in the maturational changes characteristic of the developing neonatal small intestine (9). In the rat, the major circulating glucocorticoid hormone, corticosterone, increases during the suckling period and reaches highest levels at weaning (17). We have shown that in the neonatal rat, administration of the synthetic glucocorticoid hormone Dex results in the precocious development of CRIP mRNA and protein levels. This increase could be the result of either a glucocorticoid-stimulated increase in CRIP gene expression within existing intestinal cells or a precocious maturation of epithelial cells replacing less mature cells. Increased CRIP mRNA levels could also be the result of a glucocorticoid-mediated decrease in mRNA turnover. Regardless of mechanism, the effect of Dex on CRIP mRNA appears to be a direct one, as we have also observed a progressive increase (to 60%) in CRIP mRNA in cultured intestinal epithelial cells

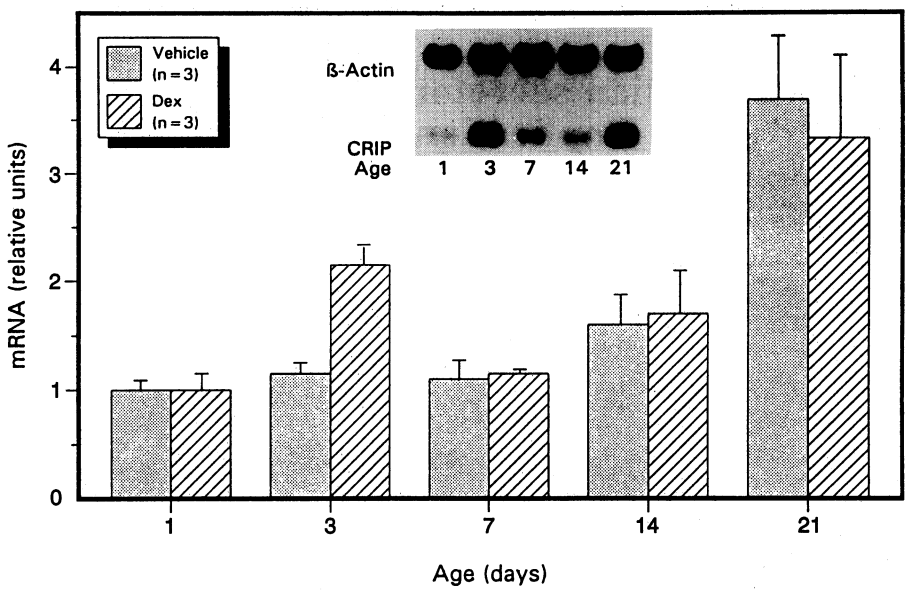

FIG. 6. Effect of Dex on developmental expression of CRIP mRNA. Pups were treated with Dex at 1 mg/kg or vehicle on postnatal days 1 and 2. Bar graph shows CRIP mRNA levels (relative to levels on day 1) in pups on postnatal days 1, 3, 7, 14, and 21 (n = 3, mean ± SEM). Relative mRNA levels were determined by densitometry of autoradiographs. (*Inset*) Representative Northern analysis of intestinal RNA (15 μg/lane) from Dex-treated animals.

(IEC-6) (18) after incubation with up to 5 μM Dex (data not shown).

For other developmentally regulated intestinal proteins, such as sucrase, there is evidence supporting the theory that glucocorticoids result in the precocious maturation of the intestine (9). Glucocorticoids stimulate an increase in sucrase mRNA levels and enzyme activity when mature sucrase-producing cells replace less mature cell populations (19). Thus, the transient rise in CRIP mRNA seen in the Dex-treated neonatal intestine could be due, in part, to a general glucocorticoid-stimulated precocious development of the small intestine. This result is supported by the fact that we were unable to significantly increase CRIP mRNA levels in the adult rat small intestine by Dex administration.

The present experiments suggest that, in the developing neonatal rat, CRIP is under the control of glucocorticoid hormones. This report demonstrates the glucocorticoid regulation of CRIP and gives an example of a hormonally regulated LIM motif-containing protein.

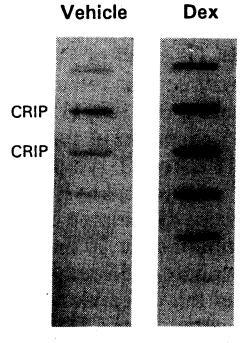

FIG. 7. Effect of Dex on ^{65}Zn binding to CRIP. Pups were treated with Dex at 1 mg/kg or vehicle on postnatal days 1 and 2. On day 3, intestinal cytosolic constituents were separated by Superdex 75 HR gel-filtration HPLC. Chromatographic fractions (0.5 ml) numbered 30–36 were applied by slot blot to nitrocellulose and analyzed by ^{65}Zn blot assay for CRIP as described (2). CRIP is eluted predominately in fractions 31 and 32.

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